STIMULATION OF MURINE B LYMPHOCYTES BY ISOLATED C3b*

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The nature and sequence of events leading to stimulation of B lymphocytes and to clonal proliferation and differentiation of antibody-secreting cells are not yet known. One of the proposed mechanisms involves the action of complement on C3b receptor-bearing B lymphocytes (1). In these experiments we investigated the effect of isolated C3b on DNA synthesis and blast cell formation in mouse lymphocyte cultures. This approach was taken since C3b is an activator of the alternate complement pathway (2, 3) and when bound to C3b receptors could effect receptor-bearing cells through complement activation.

The first suggestion for complement involvement during the induction of a humoral immune response was provided by the observation that, in vivo, reduction of serum C3 levels by cobra venom factor inhibited the development of thymus-dependent humoral antibody formation (4). The role of C3 in the thymus-dependent antibody response was explained in terms of receptor-bound C3 augmenting cell cooperation and antigen presentation (5). Other investigators showed that, in vitro, T-cell mediated or mitogen-triggered antibody formation could only be elicited in the presence of C3-sufficient serum (6, 7). Furthermore, activation of the alternative complement pathway by cobra venom factor could substitute for T cells in the immune response to thymus-dependent antigens. The authors assumed that this effect was due to direct interaction of "activated" C3 with the C3b receptor of B lymphocytes.

In this report we demonstrate stimulation of DNA synthesis and blast formation in murine lymphocyte cultures by C3b. The results suggest that the stimulation is caused by activation of the alternative complement pathway by receptor-bound C3b.

Materials and Methods

Mice. DBA/2 and BDF_1 mice were bred in our colony. Male C3H/HeJ and C3HeB/FeJ mice were bought from Jackson Laboratories (Bar Harbor, Maine). Congenitally athymic mice (nu/nu) were kindly provided by Dr. J. Watson (The Salk Institute, San Diego, Calif.).

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Lymph Node Cell Suspensions. The mice were sacrificed and the spleens or mesenterial or inguinal lymph nodes aseptically removed. All suspensions were prepared by teasing the spleens or nodes through a fine mesh stainless screen into Hanks balanced salt solution (BSS).[1] The cells were washed twice in BSS and resuspended to the defined cell concentration in Eagle's minimal essential medium (MEM) (Microbiological Associates, Bethesda, Md., no. 12-126). MEM was fortified with nonessential amino acids, Na-pyruvate, L-glutamine, streptomycin, and penicillin (8). 2-mercaptoethanol (10^{-4} M) or L-cysteine (10^{-3} M) was present in the medium.

Cell Cultures. 0.4 ml of the cell suspension and 0.1 ml BSS (including fetal calf serum, C3b, or other additions) were placed into plastic petri dishes (Linbro Trays FB-16-24-TC, Linbro Scientific Co., New Haven, Conn.). The trays were incubated at 37°C in an atmosphere of 10% CO_2, 7% O_2 and 83% N_2, without rocking. 4 h before harvesting 1 μCi [^{3}H]thymidine ([*methyl*-^{3}H]thymidine, 5 Ci/mmol, from Radiochemical Centre, Amersham, England) was added. At the end of the incubation period the cells were scrubbed from the bottom of the petri dish, filtered through glass fiber filters (Whatman GF/A), washed with BSS, 5% trichloroacetic acid, and methanol. 5 ml of counting fluid (5 g 2,5-diphenyloxazole in 1 liter toluene) was added to the dried filters before counting the acid-insoluble radioactivity in a scintillation counter.

Human C3 and Its Physiologic Fragments. Human C3 was prepared from normal human serum according to the method of Nilsson and Müller-Eberhard (9). C3b was obtained from isolated C3 by trypsin treatment (10) and separated from C3a by pevikon block electrophoresis of the trypsinized material. C3 and C3b were stored at a concentration of 1 mg/ml in phosphate-buffered saline (PBS) at −70°C. Before use, the solutions were freed of aggregated material by centrifugation (100,000 *g*, 1 h), and filtered through Millipore filters (0.45 μm pore size). C3a, purified from inulin-activated human serum (11) was a gift of Dr. T. Hugli (Scripps Clinic and Research Foundation, La Jolla, Calif.). C3c was obtained by trypsin digestion of C3 and separated from residual C3b in the digest by gel filtration on Sephadex G-200 (10).

Autoradiography and Fluorescent Staining of the Cultured Lymphocytes. 3 days after the start of the culture the cells were pulsed for 4 h with [^{3}H]thymidine and harvested. The cell suspensions (0.5 ml) were layered on 5 ml of fetal calf serum (FCS) and centrifuged for 20 min at 1,500 *g*. After removal of the supernate the cell pellet was resuspended in 0.1 ml FCS and smears were prepared. For autoradiography the air-dried slides were fixed in methanol, overlayed with Kodak NTB2 emulsion, and incubated for 1 wk. For the demonstration of immunoglobulins in the cells the air-dried slides were fixed in 95% cold ethanol and sequentially incubated with rabbit antimouse Ig and fluorescent goat antirabbit IgG. The smears were observed using an Ortholux II microscope equipped for reflected light fluorescence (E. Leitz, Inc., Rochleigh, N.J.).

FCS. Fetal calf serum from Biocult Laboratories (batches 188 and 194).

LPS. Bacterial lipopolysaccharides (LPS), purified from *Salmonella minnesota* and *Salmonella abortus equi* were gifts of Dr. O. Lüderitz (Max Planck Institut für Immunobiologie, Freiburg, Germany). The LPS solution was prepared freshly for each experiment.[2]

Results

Stimulation of Mouse Lymph Node Cells by Human C3b. In order to assess a possible mitogenic effect of C3b on murine lymphocytes, mesenterial lymph node cells of BDF_1 mice were cultured for 48 h in medium without C3b or in medium containing 50 μg C3b. 4 h before harvesting, [^{3}H]TdR (1 μCi) was added to each culture and the acid-insoluble radioactivity was measured and taken as indicator for DNA synthesis of the cultured cells. The results of these experiments are depicted in Table I. In the absence of C3b only marginal amounts of TdR were incorporated, while addition of 50 μg of isolated C3b had a definite stimulatory effect. The presence of 3.2% FCS in the medium—which by itself

[1] *Abbreviations used in this paper:* BSS, Hanks balanced salt solution; FCS, fetal calf serum; LPS, bacterial lipopolysaccharide (endotoxin); 2-ME, 2-mercaptoethanol; PBS, phosphate-buffered saline; TdR, thymidine.

[2] Hartmann, K.-U., and S. Greissel. Manuscript in preparation.

stimulates DNA synthesis in these lymphocyte cultures—enhances the stimulatory effect of C3b. The degree of stimulation by C3b and FCS was partially dependent on the cell concentration in the cultures. At 1.6×10^6 cells/culture the C3b-induced increase in DNA synthesis was approximately 10-fold.

The stimulation of TdR incorporation was dependent on the amounts of C3b present in the cultures (Fig. 1). In these experiments 6 μg of C3b per 0.5 ml cultures already resulted in detectable stimulation. It appears that 50 μg C3b per culture did not yet yield maximal stimulation. As mentioned above, the stimulation of DNA synthesis by C3b is potentiated in the presence of FCS. The

TABLE I
Stimulation of DNA Synthesis of Mouse Lymph Node Cells by C3b

BDF1 lymph node cells	FCS	Ipm/culture	
		Without C3b	+50 μg C3b
	%		
1.6×10^6/culture	—	570	5,230
"	3.2	9,800	21,200
0.8×10^6/culture	—	490	2,540
"	3.2	5,990	9,690
0.4×10^6/culture	—	440	1,030
"	3.2	2,470	3,840
0.2×10^6/culture	—	410	410
"	3.2	620	790

BDF_1 lymph node cells in 0.5 ml medium containing 10^{-4} M 2-ME. 3.2% FCS and 50 μg C3b (prep. 19) were added as indicated. Incubation time 48 h. [^{3}H]TdR incorporation, 4 h (exp. 855).

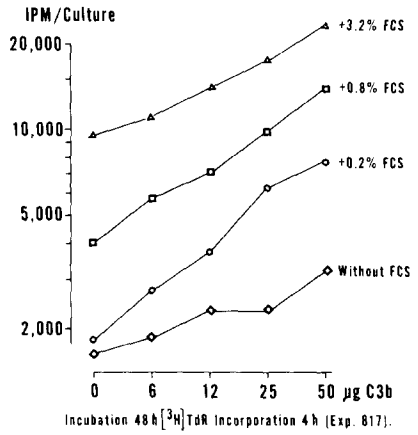

FIG. 1. Dependence of [^{3}H]TdR incorporation on C3b concentration. 1×10^6 C3HeB lymph node cells in 0.5 ml were incubated for 48 h. All the cultures contained 10^{-3} M L-cysteine. FCS and isolated C3b diluted in BSS was added as indicated. [^{3}H]TdR incorporation, 4 h (exp. 817).

results suggest that C3b and FCS act synergistically during the events stimulating DNA synthesis. This synergistic effect could be observed with as little as 0.2% FCS, corresponding to approximately 1 μl of FCS per culture. The factor or factors in FCS responsible for the stimulation of DNA synthesis and for the cooperative effect with C3b are heat stable, since heating of FCS for 30 min at 56°C did not diminish stimulatory activity. Dialysis of FCS against 100 vol BSS partially reduced its mitogenic and potentiating activity.

Time-Dependence of the Stimulatory Effect of C3b. In these experiments the stimulation of DNA synthesis by C3b was measured in lymph node cell cultures at time intervals of 24 h (Fig. 2). All cultures contained 3.2% FCS. 4 h before harvesting the cultures were pulsed with 1 μCi [^{3}H]TdR. Maximum [^{3}H]TdR incorporation in cultures containing varying amounts of C3b is only reached after 96 h incubation. This time-course shows the same kinetics as the stimulation in the presence of FCS alone, but is distinct from the stimulation by LPS which reaches its maximum after 72 h and declines thereafter.[2]

In order to obtain optimal TdR incorporation C3b had to be added at the start of the cultures. Postponement of the addition of C3b resulted in reduced TdR incorporation. In an experiment depicted in Table II, 25 μg of C3b were added at the beginning or after 24 and 48 h in culture, respectively. All cultures contained 3.2% FCS. Cultures were harvested at 24-h intervals.

Stimulation of Mouse Lymph Node Cells by Human C3. Addition of isolated C3 to lymph node cell cultures also resulted in increased TdR incorporation. When compared on a weight basis C3 was less active than C3b (Fig. 3). Treatment of the C3 preparations with 0.015 M hydrazine hydrate for 60 min at 37°C, which destroyed C3 hemolytic activity, did not affect their capacity to stimulate TdR incorporation. It should be noted however, that the C3 preparations used in these studies contained small amounts of C3b which could account for the observed stimulation. Hydrazine hydrate treatment of C3b has no effect on its activity in the alternative complement pathway (2). C3a and C3c at a molar concentration comparable to that of C3b did not stimulate TdR incorporation (Table III).

Stimulation of Lymph Node Cells of Different Strains of Mice. Stimulation

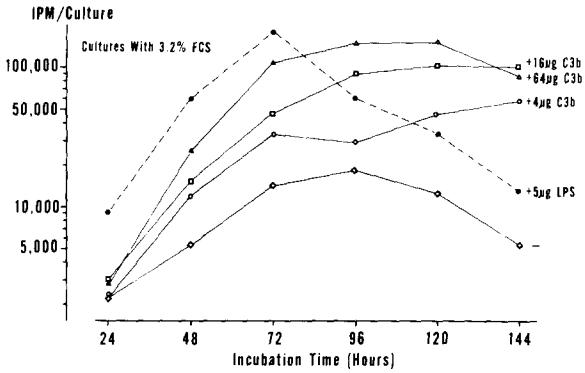

FIG. 2. Time-course of [^{3}H]TdR incorporation. 1 × 10^6 C3HeB lymph node cells in 0.5 ml cultures, containing 3.2% FCS and 10^{-4} M 2-ME. Addition of C3b and LPS as indicated; cells harvested after 24–144 h of incubation. [^{3}H]TdR incorporation, 4 h (exp. 829).

TABLE II
Effect of Delayed Addition of C3b to Lymph Node Cell Cultures On DNA Synthesis

Addition of C3b	Ipm/culture			
	48 h	72 h	96 h	120 h
—	12,000	18,000	27,600	7,400
At the start of culture	20,700	35,300	95,100	20,100
After 24 h of culture	15,300	28,300	55,400	20,900
After 48 h of culture	—	22,400	10,920	1,270

0.5 ml cultures of C3HeB/FeJ lymph node cells, containing 3.2% FCS and 10^{-4} M 2-ME. Addition of 25 μg C3b at the start or 24 and 48 h after the start of the culture. Cultures were harvested after 48, 72, 96, and 120 h of incubation. [^{3}H]TdR incorporation 4 h (exp. 832).

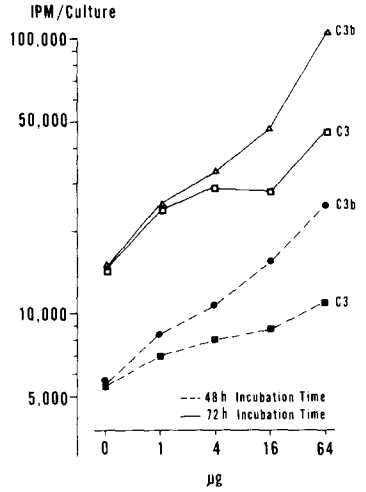

FIG. 3. Comparison of the stimulatory effect of C3b and C3. 1×10^6 C3HeB lymph node cells in 0.5 ml cultures containing 3.2% FCS and 10^{-4} M 2-ME. C3b and C3 diluted in BSS was added as indicated. Cells were harvested after 48 h (dotted lines) and 72 h (solid lines). [^{3}H]TdR incorporation, 4 h (exp. 830).

of DNA synthesis by C3b was observed in lymphocyte cultures from all strains of mice tested, which included DBA/2, C3HeB/FeJ, C3H/HeJ, and congenitally thymus-deficient nu/nu (Table IV). C3H/HeJ lymph node cells which are poor responders to the mitogenic effect of LPS (12, footnote 2) are stimulated by C3b to the same degree as lymph node cells of the other strains. The observation that lymph node cells of nu/nu mice and the nonadherent cells of normal lymph node and spleen suspensions—which are partially deprived of macrophages (not shown in the table)—were stimulated by C3b suggests that B lymphocytes are the main target of the stimulation.

Stimulation of B Lymphocytes by C3b. The number of cells exhibiting increased TdR incorporation was measured by autoradiography. Lymphocyte cultures incubated with C3b in the presence of FCS for 3 days showed 10–20%

TABLE III
Effect of C3a and C3c on DNA Synthesis of Mouse Lymph Node Cells

Addition of:	Ipm/culture	
	48 h	72 h
—	6,210	19,000
1 μg C3a	4,500	13,200
5 μg C3a	1,860	7,600
12 μg C3c	5,700	15,700
50 μg C3c	4,030	4,090
50 μg C3b	13,800	49,500

1×10^6 C3HeB lymph node cells in 0.5-ml cultures containing 3.2% FCS and 10^{-4} M 2-ME. Incubation period 48 and 72 h, respectively. [^{3}H]TdR incorporation 4 h (exp. 858 and 883).

TABLE IV
Stimulation of Lymph Node Cells of Different Strains of Mice

Lymph node cells	FCS	Ipm/culture	
		Without added C3b	+ 40 μg C3b
	%		
1.5×10^6 cells DBA/2	—	1,600	14,300
"	10	8,710	41,600
1.3×10^6 cells C3HeB/FeJ	—	1,100	24,700
"	10	5,610	74,700
1.2×10^6 cells C3H/HeJ	—	770	19,600
"	10	2,230	65,500
			+ 50 μg C3b
0.8×10^6 cells nu/nu	—	3,540	15,100
"	3.2	15,800	45,700

0.5 ml cultures containing 10^{-3} M L-cysteine. Addition of C3b (prep. 20) and FCS as indicated. Incubation period 48 h. [^{3}H]TdR incorporation 4 h (exp. 738 and 832).

enlarged blastlike cells most of which were heavily stained with silver grains when analyzed on autoradiography indicating TdR incorporation (Fig. 4 A). None of the small lymphocytes were stained by this method. In parallel experiments cell smears of the same cultures were sequentially incubated with rabbit antimouse Ig and fluorescent goat antirabbit IgG serum. Most of the blast cells were brightly fluorescent providing further evidence for the B-cell origin of the stimulated cells (Fig. 4 B).

Discussion

This study was undertaken to investigate the effect of C3b and C3 on activation of murine lymphocytes in culture. Activation was assessed by measuring

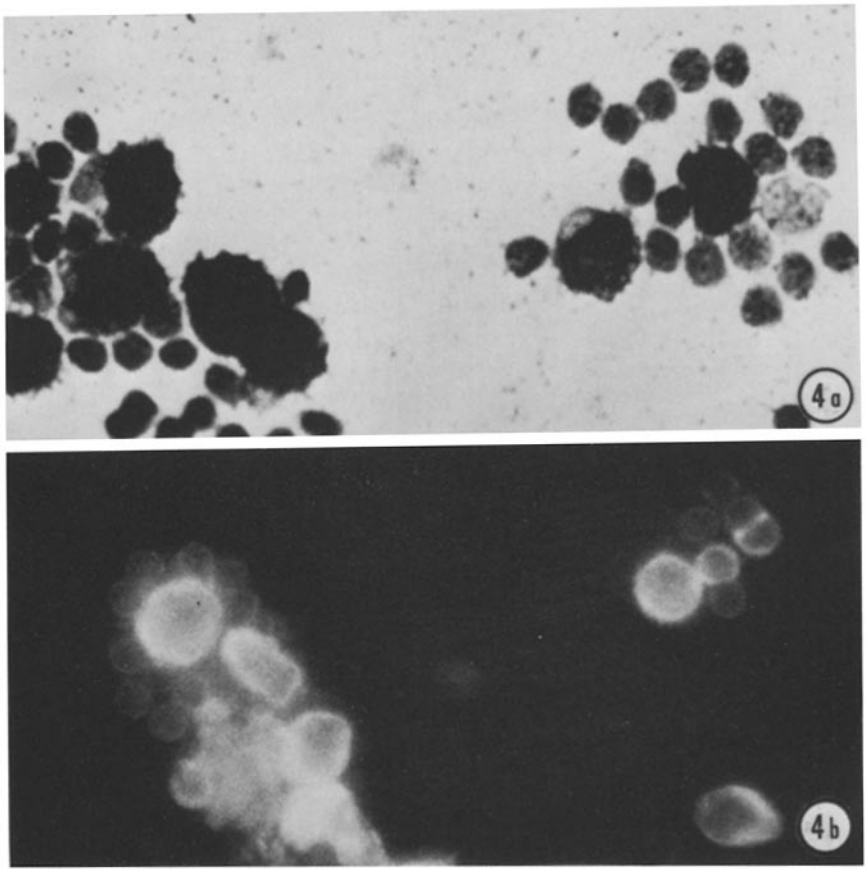

FIG. 4. Autoradiography and immunofluorescent staining of cell smears from lymphocyte cultures. 1×10^6 C3HeB lymph node cells were incubated for 72 h. The cultures contained 50 μg C3b, 3.2% FCS and 10^{-4} M 2-ME. [^{3}H]TdR incorporation, 4 h. The harvested cells were centrifuged (20 min at 1,500 *g*) through FCS and smeared on slides. The cells were stained with hematoxylin after autoradiography (4 A) or with techniques using rabbit antimouse Ig and fluorescent goat antirabbit IgG (4 B).

the increase of thymidine incorporation into DNA and by observing the development of large blastlike cells. In these experiments we used human C3 and its physiologic fragments since they are immunochemically well-defined (10, 13) and are available in larger quantities than murine components. The results show that addition of isolated C3b to mouse lymphoid cell suspensions stimulates DNA synthesis and blast cell formation. The stimulation of DNA synthesis is dose-dependent, can already be observed after 48 h of culture and reaches its maximum between 96 and 120 h.

Several preparations of C3 which served as source for the production and isolation of C3b also exhibited a stimulatory effect. When compared on a weight basis C3 was considerably less stimulatory than C3b. These results could be explained by small amounts of C3b in the C3 preparations, or by conversion of receptor-bound C3 to C3b by proteolytic enzymes present in the culture medium. C3a, the low molecular weight fragment of C3, which possesses anaphylatoxin

and chemotactic activity, did not stimulate the lymphocyte cultures, and neither did C3c, the 140,000 dalton fragment that occurs after enzymatic cleavage of C3b (10, 13). The different C3b and C3 preparations which stimulated lymphocyte cultures were free of other serum proteins as evidenced by analysis on polyacrylamide gel electrophoresis and immune electrophoresis using potent rabbit antisera to whole human serum. Significant contamination of the C3b and C3 preparations by endotoxins (LPS), which are known B-cell mitogens, could be excluded since the increase of thymidine incorporation induced by C3b follows different kinetics than thymidine incorporation induced by LPS, and secondly, since lymphoid cells from C3H/HeJ mice which respond poorly to LPS (12, footnote 2) were stimulated by C3b to the same degree as lymphoid cells of other strains of mice.

The stimulatory effect of C3b and C3 was observed in cultures without FCS and cultures supplemented with different concentrations of FCS. While the presence of FCS by itself leads to lymphocyte stimulation, it greatly potentiates the stimulatory effect of C3b or C3. This potentiation of the effect of C3b can already be observed with very low concentrations of FCS in the culture medium (Fig. 1). Heating at 56°C for 60 min did not abolish the potentiating effect, whereas extensive dialysis reduced the activity of FCS, suggesting that heat-stable and low molecular weight factors of the FCS are responsible for the synergistic activity.

Stimulation of DNA synthesis induced by C3b was observed in cultures prepared from cell suspensions of peritoneal, inguinal, and axillary lymph nodes and spleens of all the mouse strains tested. Peripheral blood lymphocytes prepared by density centrifugation on Ficoll-Hypaque could not be stimulated in our cultures by C3b and FCS, although they responded normally to phytohemagglutinin stimulation. Stimulation of DNA synthesis induced by C3b could be observed in lymph node cell cultures deprived of most of the rapidly adhering cells, and also in lymph node and spleen cell cultures from congenitally thymus-deficient (nu/nu) mice. As a result of the stimulation a great number of large blastlike cells appear after 2 to 3 days of culture; these cells have incorporated the labeled thymidine as demonstrated on autoradiography and contain immunoglobulins as shown by staining with fluorescent labeled antimouse Ig. Moreover, recent experiments showed that addition of C3b to mouse lymph node and spleen cell cultures induced the development of large numbers of anti-TNP plaque-forming cells within 48 h of incubation.[3] Taken together all these results suggest that the B lymphocyte is the main target of the stimulation induced by C3b.

The events leading to the activation of B lymphocytes by soluble C3b or C3 are not yet characterized. In the light of our present knowledge one may invision two different mechanisms leading to lymphocyte stimulation. The first mechanism involves cross-linking of C3 receptors on one cell or between different cells by C3b or C3. C3b and C3 molecules possess two stable binding sites which specifically interact with the C3b and C3d receptors, respectively (14). Since both types of receptors are present on mouse lymphocytes as shown by rosette

[3] Hartmann, K.-U., and V. A. Bokisch. Manuscript in preparation.

formation with sensitized sheep erythrocytes bearing guinea pig complement, $EAC1423b^{gp}$ (6), and EA-bearing mouse complement, $EAC1423^{m}$ (15), it is conceivable that C3b and C3 may cross-link receptors on cell surfaces. By the same mechanism C3b and C3 may also mediate contact between receptor-bearing cells. Although mouse B lymphocytes do not form rosettes with EAC1423b (15), we could demonstrate binding of C3b and C3 to 10–15% of mouse lymph node lymphocytes by indirect fluorescent techniques using rabbit antihuman C3 and fluorescent goat antirabbit IgG. Cross-linking of C3 receptors has been postulated as a mechanism for the induction of lymphokine secretion by guinea pig B lymphocytes (16). In our experiments heat-aggregated C3b was not more efficient than the monomeric form in B-lymphocyte stimulation. This observation, together with the fact that C3b is considerably more active than C3 and that FCS potentiates the C3b effect, seems to argue against cross-linking of receptors as an important event leading to stimulation of B lymphocytes.

The other mechanism concerns the role of C3b as activator of the alternative complement pathway (2, 3). C3b forms a complex with factor B (C3 proactivator) in the presence of factor D (C3 proactivator convertase) which is endowed with enzymatic activity, cleaves C3, and initiates activation of C5-9. That receptor-bound C3b can activate the alternative complement pathway has been shown with C3b-bearing Raji cells (cultured Burkitt lymphoma cells) which lysed in the presence of fresh human serum (17). The fact that in our experiments, heat treatment of FCS, which inactivates factor B, did not reduce the potentiating effect of FCS on C3b-induced stimulation does not argue against the participation of the alternative complement pathway in this reaction since it has been observed that mouse B lymphocytes can supply factor B.[4] The reduction of the potentiating effect of FCS after extensive dialysis may indicate a partial loss during dialysis of the low molecular weight factor D. It is, therefore, conceivable that C3b bound to receptors on mouse B lymphocytes in the presence of factor D initiates the formation of the $\overline{C3bB}$ complex. The function of the complement receptor in this reaction would consist in binding the enzymatically active complex which may induce activation of C3 and possibly of later acting complement components in close proximity of the lymphocyte membrane.

Summary

Addition of isolated C3b to murine lymph node cell cultures induced increased DNA synthesis. The stimulation is dependent on the dose of C3b added and is potentiated by fetal calf serum present in the medium. Isolated C3 is less stimulatory than C3b; C3a and C3c had no effect on DNA synthesis in these cultures. 10–20% large immunoglobulin containing blastlike cells developed in lymph node cell cultures stimulated by 50 μg C3b in the presence of 3% fetal calf serum. The stimulation by C3b was observed in cultures of lymph node and spleen cells of several mouse strains including C3H/HeJ and congenitally thymus-deficient (nu/nu) mice. The results suggest that B lymphocytes are the main target of the stimulatory effect of C3b. Two mechanisms which may be

[4] Halbwachs, L., and P. J. Lachmann. Reported at Meeting of the British Society of Immunology, October, 1974, London, England.

involved in the stimulation of lymphocytes by C3b are discussed: (*a*) cross-linking of receptors on the cell surface or between cells, and (*b*) the binding of C3b to receptors of B lymphocytes and the formation of the complement enzyme $C\overline{3b}\overline{B}$. The results are compatible with the suggestion that activation of C3 is part of the events triggering the B lymphocytes.

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